Clonality - X Chromosome Inactivation Assay

This method was successful in our lab using prostate tissue and for our specific objectives. Investigators must be aware that they will need to tailor the following protocol for their own research objectives and tissue under study.

Investigators can utilize X chromosome inactivation (methylation) to determine the clonality status of a tumor or premalignant lesion in females. The technique is based on a methylation-sensitive restriction enzyme and analysis of a polymorphic locus on the X chromosome. Clonal cell populations will show "loss" of the non-methylated allele after restriction digest. The assay can be performed on DNA recovered from microdissected samples. Both frozen tissue and fixed-embedded archival tissue can be utilized.

1. Materials

1. DNA sample (see Processing of Microdissected Tissue - DNA-based Analysis)
2. Proteinase K (Sigma)
3. Proteinase K buffer (0.05 M Tris-HCL, 0.001 M EDTA, 1% Tween 20, 0.1 mg/ml proteinase K, pH 8.0)
4. Phenol:chloroform:isoamyl alcohol (1:1:1, v:v:v) (Gibco)
5. 3 M Na Acetate, pH 5.2 (Life Technologies)
6. Isopropanol
7. Glycogen, 10 mg/ml (GenHunter)
8. 70% ethanol
9. Buffer 1 (50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, 50 mM NaCl)
10. Hha I restriction enzyme (Life Technologies)
11. REact 2 Buffer (Life Technologies)
12. 10X PCR buffer (100 mM Tris-HCL pH 8.3, 500 mM KCL, 15 mM MgCl2, 0,01 % w/v gelatin, autoclaved) (Perkin Elmer)
13. dNTP, each 10 mM (Perkin Elmer)
14. 7-deaza dGTP, 10 mM (Boehringer Mannheim)
15. DMSO, minimum 99.5%, GC (Sigma)
16. Forward and reverse primers, each 20 µM (Human Androgen Receptor Gene (HUMARA) CAG trinucleotide repeat microsatellite primers are described in Allen et al)
17. AmpliTaq Gold, 5 U/ml (Perkin Elmer)

18. α ^{32}P-dCTP, 20 µCi/µl (NEN)

19. Gel-Mix 6 (Life Technologies)

20. 10X TBE (1 M Tris, 0.9 M Boric Acid, and 0.01 M EDTA) (Life Technologies)

21. Loading dye (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol)

2. Methods

TIP: Investigators must be especially careful when using this methodology to analyze archival tissue specimens. Formalin-fixation in particular results in DNA that is difficult to amplify and often produces inconsistent PCR results, including artifactual allelic loss and poor amplification of large products. If this technique is utilized for analysis of archival samples, we highly recommend that replicate experiments (multiple independent dissections, triplicate PCR reactions, etc.) be used to verify results.

A: LCM and Proteinase K Treatment

1. Obtain microdissected cells using the LCM procedure.

   TIP: The number of cells needed to successfully perform the assay varies depending on the quality and processing conditions of the tissue samples. One thousand cells is recommended as a good starting point.

2. Suspend approximately 1000 microdissected cells in 20 µl proteinase K buffer.

3. Incubate overnight at 37°C.

B: DNA Purification

1. Lyse the cells overnight at 42°C, in 50 µl phenol:chloroform:isoamyl alcohol.

2. Boil 10 min at 95°C.

3. Vortex 1 min.

4. Centrifuge 20 min at 14,000 rpm at RT.

5. Pipet upper phase into new tube and discard lower phase.

6. Add 5 µl 3M NaAc.

7. Add 250 µl isopropanol.

8. Add 2 µl glycogen.

9. Vortex briefly.

10. Place on dry ice for 30 min.

11. Centrifuge 14,000 rpm for 30 min.
12. Add 300 µl 70% ethanol.
13. Vortex briefly.
14. Centrifuge 2 min at 14,000 rpm at RT.
15. Discard supernatant.
16. Dry pellet at RT for 5 min, or 37°C for 2 min.
17. Resuspend the pellet in 20 µl buffer 1.
18. Incubate at 37°C for 3 min to completely dissolve the pellet.

C: DNA Digestion

1. Pipet 8 µl of the resuspended DNA into a reaction tube.
2. Add 1 µl REact buffer 2.
3. Add 1 µl Hha I.
4. Incubate overnight at 37°C.
5. Incubate at 90°C for 10 min.
6. Use 2 µl of this digested DNA for PCR (see below).
7. Use 2 µl of the non-digested DNA from B18 above as a negative control for PCR.

   TIP: Parallel analysis of control DNA that is known to be from a monoclonal population is recommended to verify the efficiency of the restriction digest. DNA recovered from a tissue specimen that was processed similar to the tissue sample under study is ideal.

D: PCR

1. Set up the following PCR reaction:

2.0 µl	DNA sample
2.0 µl	10X PCR buffer
0.4 µl	dATP
0.4 µl	dCTP
0.4 µl	dTTP

0.4 µl	7-deaza dGTP
0.4 µl	Forward primer
0.4 µl	Reverse primer
0.2 µl	AmpliTaq Gold
0.4 µl	32-P dCTP
1.0 µl	DMSO
12 µl	DEPC-H_2O
	Total volume = 20 µl

2. Cycles are as follows:

Cycles	Temp (°C)	Time (sec)
1	94	20 seconds
40 cycles	94 60 72	45 seconds 45 seconds 2 minutes
1	72	20 minutes

E: Gel Electrophoresis

1. Prepare gel consisting of 6% acrylamide as in LOH protocol.
2. Add 4 µl dye to 20 µl PCR product.
3. Denature the samples for 5 min at 94°C.
4. Load 3 µl onto gel.
5. Electrophorese at 1800 volts for 1 to 2 hours.

   TIP: In clonality analysis, the PCR products are ~200 bp. Therefore do not overload the gel with sample and run the gel for a longer period of time to sharpen the bands and increase separation.

6. Transfer the gel to 3 mm Whatman paper and dry. Perform an autoradiograph with Kodak BIOMAX film as described in LOH protocol.

   TIP: Try a short exposure (1 hour) time first and only re-expose depending on the results.

3: Results

A sample is considered to be composed of a monoclonal cell population when two alleles are recognized in the undigested DNA sample and complete absence of one allele is seen in the digested DNA sample.

TIP: DNA that is recovered from microdissected samples and "semi-purified" using a one-step proteinase K buffer strategy will sometimes produce "non-specific" PCR products in addition to the true alleles. Normal-cell DNA from archival specimens from control subjects serves as a good comparator and can assist in interpretation of allele patterns.

4: References

Allen RC, Zoghbi HY, Moseley AB, Rosenblatt HM, Belmont JW. Methylation of HpaII and HhaI sites near the polymorphic CAG repeat in the human androgen-receptor gene correlates with X chromosome inactivation. Am J Hum Genet 51:1229-39, 1992 .

Enomoto T, Fujita M, Inoue M, Tanzawa O, Nomura T, Shroyer K. Analysis of clonality by amplification of short tandem repeats. Carcinomas of the female reproductive tract. Diagnostic Molecular Pathology 3:292-7, 1994.